AL/OE-TR-1994-0072
WRAIR-TR-1994-0006

ARMSTRONG LABORATORY

RANGE-FINDING STUDY FOR A REPRODUCTIVE ASSESSMENT OF 1,3,5-TRINITROBENZENE ADMINISTERED IN THE DIET OF SPRAGUE-DAWLEY RATS

E. R. Kinkead
R. E. Wolfe
S. A. Salins
C. D. Flemming

MANTECH ENVIRONMENTAL TECHNOLOGY, INC.
P.O. BOX 31009
DAYTON, OH 45437

D. J. Caldwell
C. R. Miller

U. S. ARMY MEDICAL RESEARCH DETACHMENT, WRAIR

J. R. Latendresse

OCCUPATIONAL AND ENVIRONMENTAL HEALTH DIRECTORATE
TOXICOLOGY DIVISION, ARMSTRONG LABORATORY
WRIGHT-PATTERSON AFB OH 45433-7400

April 1994

FINAL REPORT FOR THE PERIOD NOVEMBER 1992 THROUGH MAY 1993

Approved for public release; distribution is unlimited

AIR FORCE MATERIEL COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO 45433-6573

NOTICES

When US Government drawings, specifications or other data are used for any purpose other than a definitely related Government procurement operation, the Government thereby incurs no responsibility nor any obligation whatsoever, and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

Please do not request copies of this report from the Armstrong Laboratory. Additional copies may be purchased from:

National Technical Information Service
5285 Port Royal Road
Springfield, Virginia 22161

Federal Government agencies and their contractors registered with the Defense Technical Information Center should direct requests for copies of this report to:

Defense Technical Information Center
Cameron Station
Alexandria, Virginia 22314

DISCLAIMER

This Technical Report is published as received and has not been edited by the Technical Editing Staff of the Armstrong Laboratory.

TECHNICAL REVIEW AND APPROVAL

AL/OE-TR-1994-0072

WRAIR/TR-1994-0006

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

TERRY A. CHILDRESS, Lt Col, USAF, BSC
Director, Toxicology Division
Armstrong Laboratory

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 1994	Final -November 1992 - May 1993

4. TITLE AND SUBTITLE
Range-Finding Study for a Reproductive Assessment of 1,3,5-Trinitrobenzene Administered in the Diet of Sprague-Dawley Rats

5. FUNDING NUMBERS
Contract F33615-90-C-0532
PE 62202F
PR 6302
TA 630200
WU 63020002

6. AUTHOR(S)
E.R. Kinkead, R.E. Wolfe, C.D. Flemming, D.J. Caldwell, C.R. Miller, and J.R. Latendresse

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
ManTech Environmental Technology, Inc.
P.O. Box 31009
Dayton, OH 45437-0009

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Armstrong Laboratory, Occupational and Environmental Health Directorate
Toxicology Division, Human Systems Center
Air Force Materiel Command
Wright-Patterson AFB OH 45433-7400

10. SPONSORING;MONITORING AGENCY REPORT NUMBER
AL/OE-TR-1994-0072
WRAIR/TR-1994-0006

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

The soil and groundwater of several military installations targeted for restoration contain measurable quantities of 1,3,5-trinitrobenzene (TNB). As part of the process to develop environmental and health effects criteria, a single-generation reproduction study was performed. Male and female Sprague-Dawley rats received diet containing approximately 800, 400, or 70 mg TNB/kg diet throughout the study. Mating occurred following 14 days of treatment. Dams and pups were maintained through 21-days postpartum while male rats were necropsied following 35 treatment days. No mortality occurred in parental animals during the study. A treatment-related decrease in food consumption occurred in both sexes with a concurrent depression of body weight gain. Mean testes weights of the high-and mid-dose rats were significantly less than control testes weights. Likewise, epididymides of test animals weighed significantly less than those of controls. Histopathologic examinations showed testicular degeneration in 100% of the high-dose dams and 70% of the mid-dose animals. Sperm depletion was evident in the lumen of these same groups. All high-dose dams and one mid-dose dam displayed signs of neurotoxicity, primarily head tilt and loss of equilibrium, during the postpartum time period. Histopathologic examinations of brain samples from these animals showed vacuolated neuropil in the ovilary deep cerebellar, and vestibular nuclei. Mating indices were normal, however 4- and 21-day pup survival rates were significantly less in the high-dose group of animals.

14. SUBJECT TERMS		15. NUMBER OF PAGES
1,3,5-trinitrobenzene	TNB	39
Rat	Feeding Study	16. PRICE CODE
Reproduction	Testicular Degeneration	

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

THIS PAGE INTENTIONALLY LEFT BLANK

PREFACE

This is one of a series of technical reports describing results of the experimental laboratory programs conducted at the Toxic Hazards Research Unit, ManTech Environmental Technology, Inc. This document serves as a final report on the range-finding study for a subsequent, more definitive reproduction study of 1,3,5-trinitrobenzene administered in the diet of Sprague-Dawley rats. The research described in this report began in November 1992 and was completed in May 1993 under Department of the Air Force Contract No. F33615-90-C-0532 (Study No. A02). Lt Col Terry A. Childress served as Contract Technical Monitor for the U.S. Air Force, Armstrong Laboratory. This study was sponsored by the U.S. Army under the direction of LTC Daniel J. Caldwell, USAMRD/WRAIR.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

DNB	Dinitrobenzene
EMH	Extramedullary hematopoiesis
g	Gram
kg	Kilogram
mg	Milligram
mL	Milliliter
N	Number
p	Probability
SEM	Standard error of the mean
SIDS	Screening Information Data Set
TNB	1,3,5-Trinitrobenzene
TNT	2,4,6-Trinitrotoluene

SECTION 1
INTRODUCTION

Several Army installations targeted for restoration have measurable quantities of 1,3,5-trinitrobenzene (TNB) in the soil and groundwater. 1,3,5-Trinitrobenzene is a dimorphic crystalline solid that is easily dissolved in organic solvents. It is primarily used as an explosive but has also had limited use in the vulcanization of rubber. 1,3,5-Trinitrobenzene and a similar compound, dinitrobenzene (DNB) are used to produce plastics, herbicides, and paints and can enter domestic-drinking water reservoirs via domestic effluent.

The acute toxicity of TNB has been reported by Fitzgerald et al. (1992). The acute oral toxicity of TNB suspended in corn oil was reported as 298 and 275 mg/kg in male and female rats, respectively, and >900 and 702 mg/kg in male and female mice, respectively. No deaths occurred when the neat TNB was in contact with rabbit skin for 24 h (2 g/kg limit test). 1,3,5-Trinitrobenzene was found to be a mild skin sensitizer in guinea pigs but did not cause acute irritation on rabbit skin. When applied as a powder, the treated eyes of all test rabbits received scores of severe for redness, chemosis, and opacity through 96 h posttreatment. 1,3,5-Trinitrobenzene caused irreversible damage to the ocular tissue and is considered to be corrosive.

Munition workers exposed to 2,4,6-trinitrotoluene (TNT) have developed skin irritation, liver damage, and anemia (Hathaway, 1977; Morton et al., 1976; Stewart et al., 1945). Animal studies have shown that oral treatment with structurally similar 1,3-dinitrobenzene or TNT causes anemia and increases methemoglobin concentration. Those compounds also produce hypertrophy of the liver and spleen and degeneration of the germinal epithelial lining of the seminiferous tubules resulting in decreased spermatogenesis (Cody et al, 1981; Levine et al., 1983, 1984; Furedi et al., 1984a,b). Toxicity studies of TNB following inhalation exposure were not available. Information was not available concerning developmental or reproductive toxicity induced by TNB.

Exposure to TNB can occur through contact with wastewater effluents released from facilities that synthesize or produce munitions, or from the disposal of solid TNT wastes (Ryon et al., 1984; U.S. EPA, 1989). Limited information is available on the quantity of TNB in the environment.

One objective of this range-finding study was to determine the dose levels in the diet that rodent pups could tolerate without frank toxicity when they began self-feeding during the weaning and post-weaning periods. Additionally, data from this range-finding study will be used to determine the dose levels to be used in a 90-day modified Screening Information Data Set (SIDS) protocol to address the developmental and reproductive toxicity of TNB in rats.

SECTION 2

MATERIALS AND METHODS

Test Agent and Doses

The TNB/diet mixture was provided by the U.S. Army through a contract with the Environmental Protection Agency. Pertinent chemical and physical properties of the test compound are listed below:

1,3,5-Trinitrobenzene (TNB)	
Synonyms:	Trinitrobenzene
	Benzenite
CAS #:	99-35-4
Empirical Formula:	$C_6H_3N_3O_6$
Formula Weight:	213.11
Vapor Pressure	3.2×10^{-6} mmHg at 20° C

Rat Chow. The test material was mixed in ground Purina Formulab #5002 (Ralston Purina, St. Louis, MO), certified rodent diet meal.

The TNB was administered by the oral route, mixed appropriately in the diet. The mean concentrations and (range) per target dose of five prepared diet batches per test concentration were 793 (741-837), 403 (367-424), and 66.7 (63.7-69.0) mg TNB/kg diet. The TNB-diet preparation and analyses are summarized in Appendix A.

Test Animals, Group Assignments, Clinical Measurements

Fifty male and fifty female Sprague-Dawley-derived outbred albino rats [Crl:CD®(sd)BR] known as Charles River CD rats, were purchased from Charles River Breeding Laboratories, Raleigh, NC. The rats were 14 weeks of age upon arrival and 16 weeks of age at initiation of the treatment period. All rats were identified by tail tattoo and were subjected to a two-week acclimatization period. Rodent water from a reverse-osmosis system and feed were available *ad libitum*. Animal room temperatures were maintained at 21 to 25 °C, and the light/dark cycle was set at 12-h intervals. Parental rats were single housed (except for the mating period) in clear plastic cages with hardwood-chip bedding (Bettachip®, Northeastern Products Corp., Warrensburg, NY). During the mating period, the animals were housed in clear plastic cages with stainless steel wire bottoms. There were four study groups with target doses of 0, 70, 400, and 800 mg TNB/kg diet. Rats were assigned to groups of 10 per sex by means of a computer-generated randomization, stratified by body weight such that the mean body weights of all groups were homogeneous by statistical analysis at study initiation.

Rats were observed twice daily for signs of toxic stress. Male rat body weights were measured weekly. Female body weights were measured in the same manner until confirmation of mating. During gestation, females were weighed on gestation Days 0, 4, 7, 14, and 20. Dams producing litters were weighed on Days 0, 4, 7, 14, and 21 postpartum, then weekly thereafter.

Food consumption was determined during the prebreeding period for both male and female rats. Food consumption of individual dams was measured for Gestation Days 0 to 7, 7 to 14, and 14 to 20 and for postpartum Days 0 to 7 and 7 to 14. Male food consumption was calculated weekly through study termination. Food consumption was not measured during the mating period when more than one rat was in a cage, nor during Days 14 to 21 postpartum when pups were beginning to eat from the feed jars. Feed jars were cleaned on a weekly basis at which time all leftover food was discarded. Food consumption was measured every two days until the postpartum period when it was measured daily in the female rats. The live and dead pups were counted and sexed on postpartum/lactation Day 0. All pups were counted and live pups were weighed and sexed on 1, 4, 7, 14, and 21 days after birth. Standardization of litter sizes, four per sex when possible, occurred on Day 4. Pups were examined for external abnormalities. Pup food consumption was not measured.

General Study Design

Male rats were dosed from 14 days prior to mating and throughout the mating period for a total of 35 days. Female rats were also dosed from 14 days prior to mating, during mating and gestation, postpartum (21 days), and for one-week postweaning for a total of 70 days. Pups were maintained on treated diet for a total of 7 to 14 days postweaning.

One male and one female were cohabited, selected from within their respective dose groups, beginning on Day 14 after the onset of dosing. The pairs remained cohabited until either a copulation plug was present or sperm were present in the vaginal wash for up to 5 days. If copulation did not occur by 5 days, the males were changed and the pairs observed for signs of mating for up to another 5 days. The day a copulation plug was present or sperm was found in the vaginal wash was defined as Day 0 of gestation.

Blood samples were taken from nonfasted parental animals at sacrifice. One-half of the number male animals and all of the female animals had blood removed via the vena cava at necropsy. Methemoglobin assays, measured within 1 h of blood collection, were performed using the method of Evelyn and Mallog (1938).

Activity tests were performed on the parental rats. The tests were performed on male rats postmating and prior to sacrifice; on dams during the postpartum period and again prior to sacrifice. Figure-Eight Maze and Opto-Varimex (Columbus Instruments International Corp., Columbus, OH) activity tests were performed.

Animals were placed singly into the maze, where movement was tracked via eight infrared sensors located along the path. The computer tallied the rat's activity based on the number of detectors triggered. Each animal was tracked for nine sequential 10-min sessions, for a total period of 90 min.

Four test chambers were used in the Opto-Varimex test, each having an observation area approximately 17×17.5 in., with sensors at 1-in. intervals (15 per side). Exposure sessions consisted of a single 5-min interval conducted with the room lights turned off. Following each run, the chambers were sprayed with a disinfectant/deodorant and wiped clean.

Male rats were necropsied following 35 treatment days. Sperm count and morphology were evaluated in two to three males per group at sacrifice. Sperm was removed from the right cauda epididymis and analyzed microscopically using a videomicrography system (Cell Soft Automated Semen Analyzer, Cryo Resources, Ltd.). The testes and epididymides were weighed. Bouin's fixative was used to fix the testes and epididymides. Female rats were necropsied following 70 treatment days. The spleen, liver, and kidneys were removed from representative animals and fixed in 10% buffered formalin solution. After routine processing, the tissues were embedded in paraffin and stained with hematoxylin and eosin for microscopical examination. Pups were examined for gross lesions at necropsy 7 to 14 days postweaning.

Statistical Analysis

Maternal body weights, pup weights, organ weights, organ weight ratios, food consumption, and TNB dose calculations were treated for statistical significance using a one-factorial analysis of variance with Bonferroni multiple comparisons (Rosner, 1990). A one-factorial repeated measures analysis of variance with Bonferroni multiple comparisons was used for paternal body weight (Barcikowski, 1983). Mating indices and histopathologic results were analyzed by a Chi-square test of proportions applied to the incidence data (Rosner, 1990). Tissue lesion severity data were analyzed using the Kruskal-Wallis analysis of variance (Rosner, 1990).

Sperm analysis and Opto-Varimex data were analyzed using one-way analysis of variance, employing Dunnett's technique (Sokal and Rohlf, 1981) for multiple comparisons between controls and treatments when significant differences ($p < 0.05$) occurred. Parametric analysis techniques were preferred,

but a Kruskal-Wallis rank-based analysis of variance (Sokal and Rohlf, 1981) was used when transformation techniques failed to present a normal distribution.

SECTION 3
RESULTS

General Toxicity

No mortality occurred in parental rats during the course of the study. A statistically significant decrease of mean body weight gain of both male and female rats in the high- and mid-dose groups occurred following the first week of treatment and continued through termination of the study (Figures 1 and 2). Food consumption was significantly decreased ($p < 0.01$) in the high- and mid-dose groups of rats through six days, after which food consumption returned to pretreatment levels (Figures 3 and 4). Food consumption of female rats increased during the postpartum (lactation) period compared to the premating and gestation periods. However, food consumption in the high-dose females was significantly ($p < 0.01$) less than the other groups at most time points measured. Following the mating period, male rats consumed approximately 30 g diet/day (Figure 5). This consumption rate resulted in the male rats receiving approximately 51, 23, and 3 mg TNB/kg/day in the high-, mid-, and low-dose groups, respectively (Figure 6). The female rats consumed approximately 20 g diet/day during the premating period. This increased to approximately 26 g diet/day during the gestation period and as high as 70 g diet/day during the lactation period (Figure 7). The food consumption data convert to approximately 60, 30, and 4 mg TNB/kg/day through gestation and 110, 55, and 8 during the lactation period for the high-, mid-, and low-dose groups, respectively (Figures 8 and 9).

Male and female rats receiving mid- and low-dose diet displayed circling behavior wherein they would grasp the base of their tail with their teeth and then circle. All high-dose female rats and one mid-dose female rat displayed clinical signs of toxicity during the lactation phase of the study, when food consumption and resultant TNB dose were increased. Clinical signs in these rats began with head tilt, followed by loss of equilibrium, progressing to a "cork-screw-like" motion when moving in the cage. Animals did not recover from these signs through termination of the study, although three of the high-dose rats were returned to control diet for the final two weeks of the study. No signs of toxic stress were noted in male rats during the 35-day period.

Methemoglobin assays performed on the treated parental animals at necropsy showed no increase over control values.

Except for the high-dose females, TNB-treated rats tested for activity showed no differences from control animals at any of the interim testing periods. The high-dose female rats showed a significant

increase in distance traveled and time ambulatory prior to study termination. Other parameters tested (time resting, time stereotypic, number of small movements, clockwise rotations, counter-clockwise rotations, and rearing) were not different from control values. Figure-eight maze activity measured at study termination is shown in Figure 10. There were no differences between TNB-treated and control groups.

The high-dose male rats, sacrificed following mating, showed adverse effects for all measurements of sperm function/activity (Table 1). The number and concentration of motile cells were greatly reduced in the high-dose group, with no cells traveling in circular patterns. The percent of sperm cells traveling in a circular pattern was reduced at the mid-dose group. Some apparent differences between mid-dose animals and controls are not statistically significant due to low sample size ($N=2$). Mean absolute testes weights of the high- and mid-dose rats were significantly ($p<0.01$) less than control testes weight (Table 2). Likewise, mean absolute weights of epididymides of these two groups were significantly ($p<0.05$) less than the control group value. Relative testes weight was significantly ($p<0.01$) different than the control mean for the high-dose group only.

Bilateral testicular atrophy was apparent grossly in male rats treated with the high dose of TNB. These lesions were confirmed during the histopathologic examination where 100% of the high-dose and 70% of the mid-dose rats had testicular degeneration (Table 3; Figure 11). Sperm depletion and presence of degenerated germ cells in the lumen of the epididymides at several levels (head, body, tail) strongly correlated with the degenerative changes observed in the seminiferous tubules of the testes.

Discoloration and enlargement of spleens were common findings in female rats from the high- and mid-dose groups. These observations correlated well with the hemosiderosis and extramedullary hematopoiesis (EMH) in the spleens diagnosed microscopically in these groups of rats (Table 4; Figures 12 and 13). The severity of hemosiderosis and EMH appears to be dose related. Brains from five female rats per group were examined histopathologically because of the clinical signs of neurotoxic deficit during treatment. The brain from each of two male rats per group was also examined microscopically. 1,3,5-Trinitrobenzene-induced encephalitis (Tables 3 and 4; Figures 14 and 15) was found in 5/5, 3/5, and 1/2 of the high-dose females, the mid-dose females, and the high-dose males, respectively. This lesion was found in two mid-dose female rats and one high-dose male rat that did not display clinical signs during the study. The lesions were often bilateral and most consistently located in the olivary complex of the medulla oblongata and the cerebral peduncle. Other regions where encephalitis was also detected included the inferior calyculus and the facial nuclear region.

Mating and Fertility

A copulation index of 100% was observed for all control and treated groups (Table 5). The fertility index was 90% in groups given the mid- or the low-dose diet, but was 100% in the high-dose and control groups. No significant treatment-related differences were noted in length of gestation, sex ratio, or mean number of offspring per litter. A decrease ($p < 0.05$) was apparent in the 4- and 21-day survival indices as well as the lactation index in the high-dose group only. Mean body weights of live pups in the high-dose group were statistically significantly decreased in a treatment-related manner throughout the 21-day lactation period (Figure 16). The decrease was noted as early as 4 days in both sexes of pups in the high-dose group ($p < 0.01$) and at Day 21 in the mid-dose male pup group. A difference ($p < 0.05$) was noted at Day 14 in the mid-dose male group and at Day 21 in the mid-dose female group. Ringtail was noted in one litter of the high-dose group, four litters of the mid-dose group, and one litter of the low-dose group. Ringtail was not noted in any litter of the control group animals. No other external or visceral malformations were noted in any pups at necropsy.

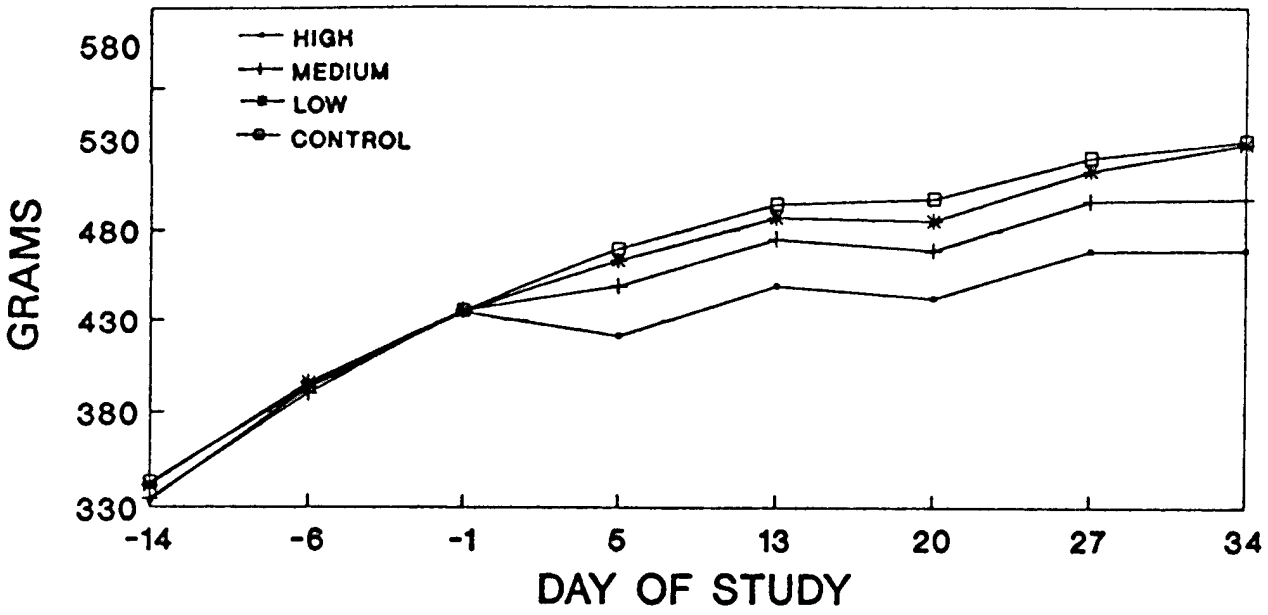


Figure 1. Mean Body Weights of Male Rats Administered TNB in Diet

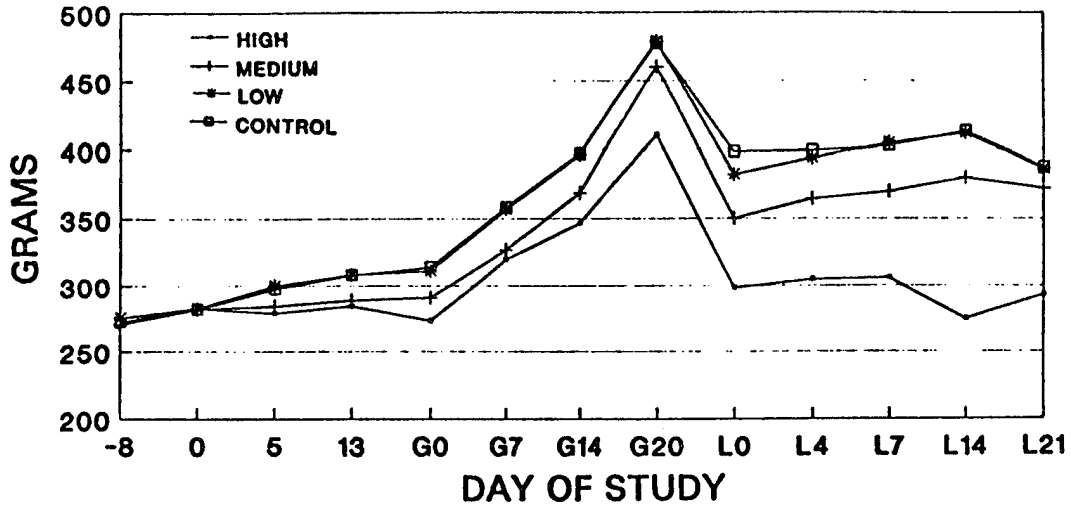


Figure 2. Mean Body Weights of Female Rats Administered TNB in Diet

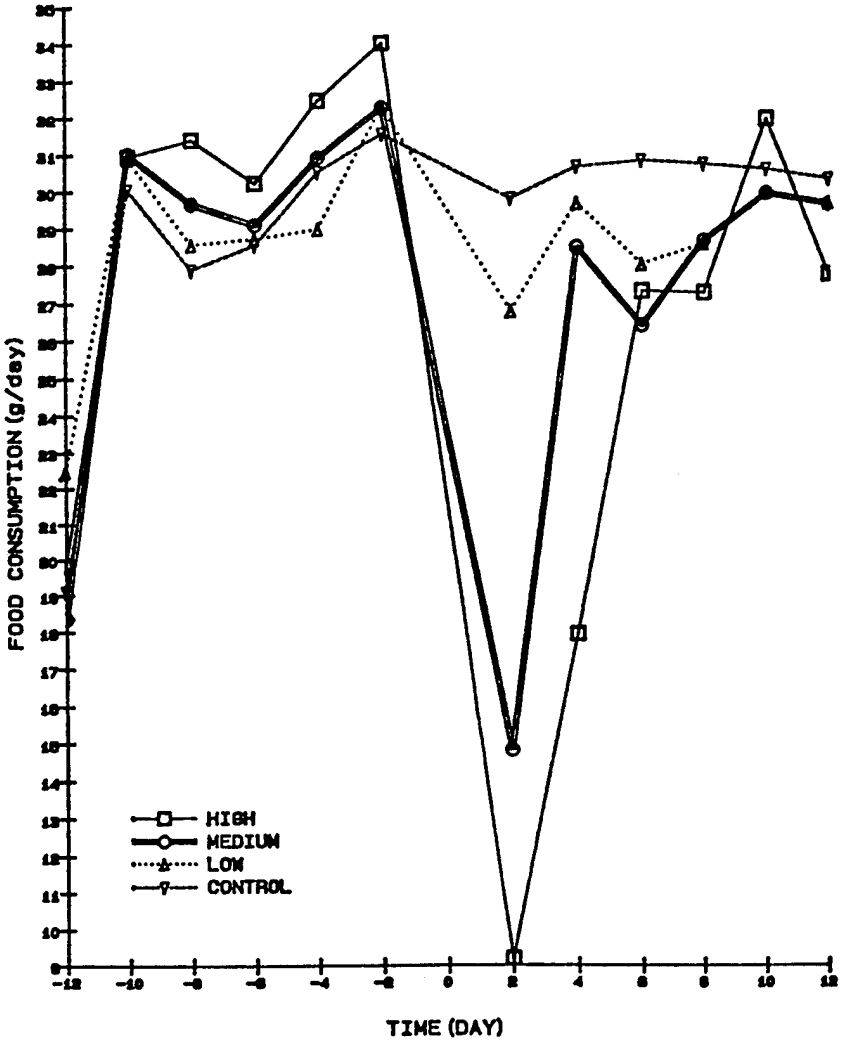


Figure 3. Mean Food Consumption of Male Rats During Two-Week Pretreatment Period and During Two Weeks Treatment Prior to Mating

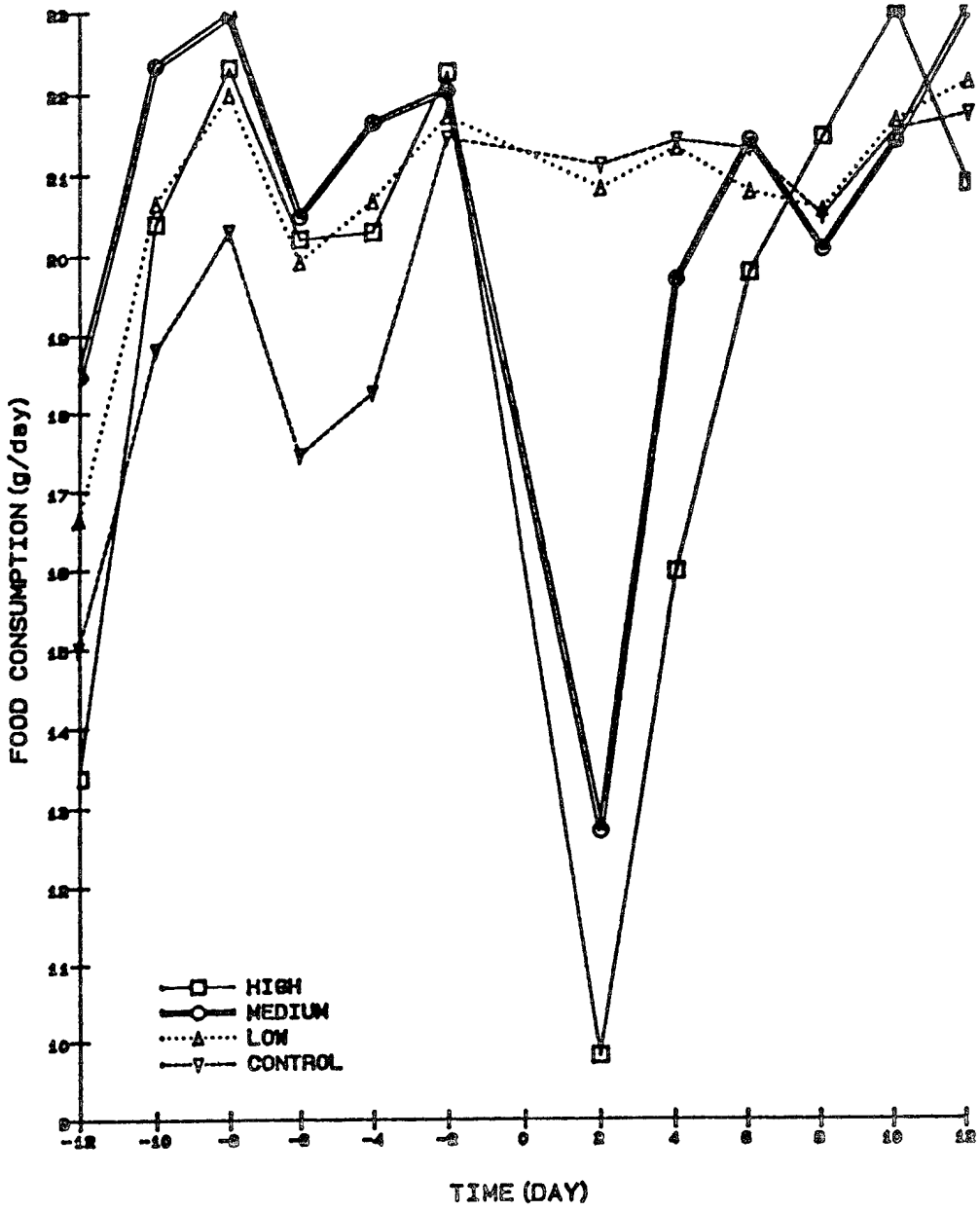


Figure 4. Mean Food Consumption of Female Rats During Two-Week Pretreatment Period and During Two Weeks Treatment Prior to Mating

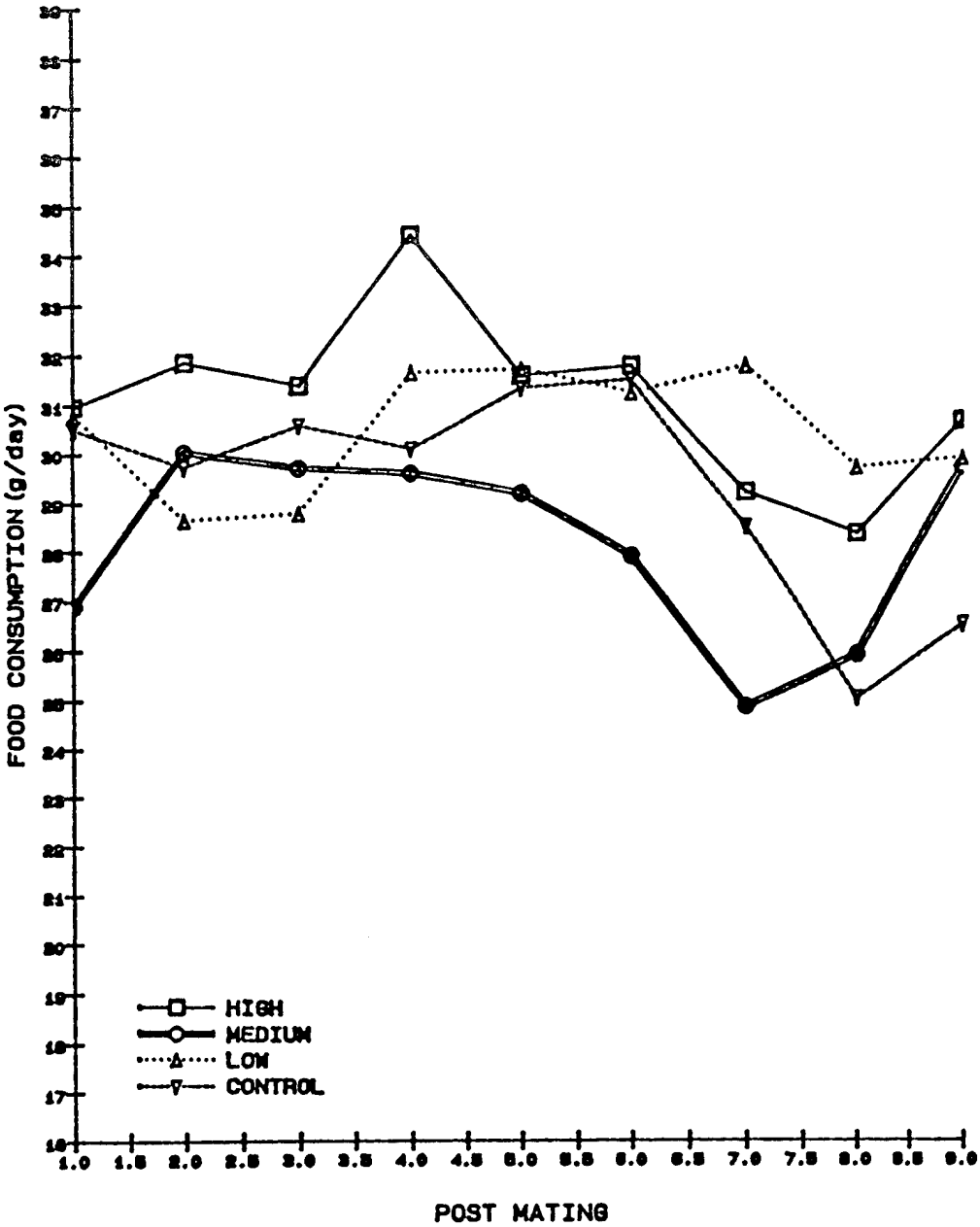


Figure 5. Mean Food Consumption of Male Rats Maintained on Treated Diet Following Mating

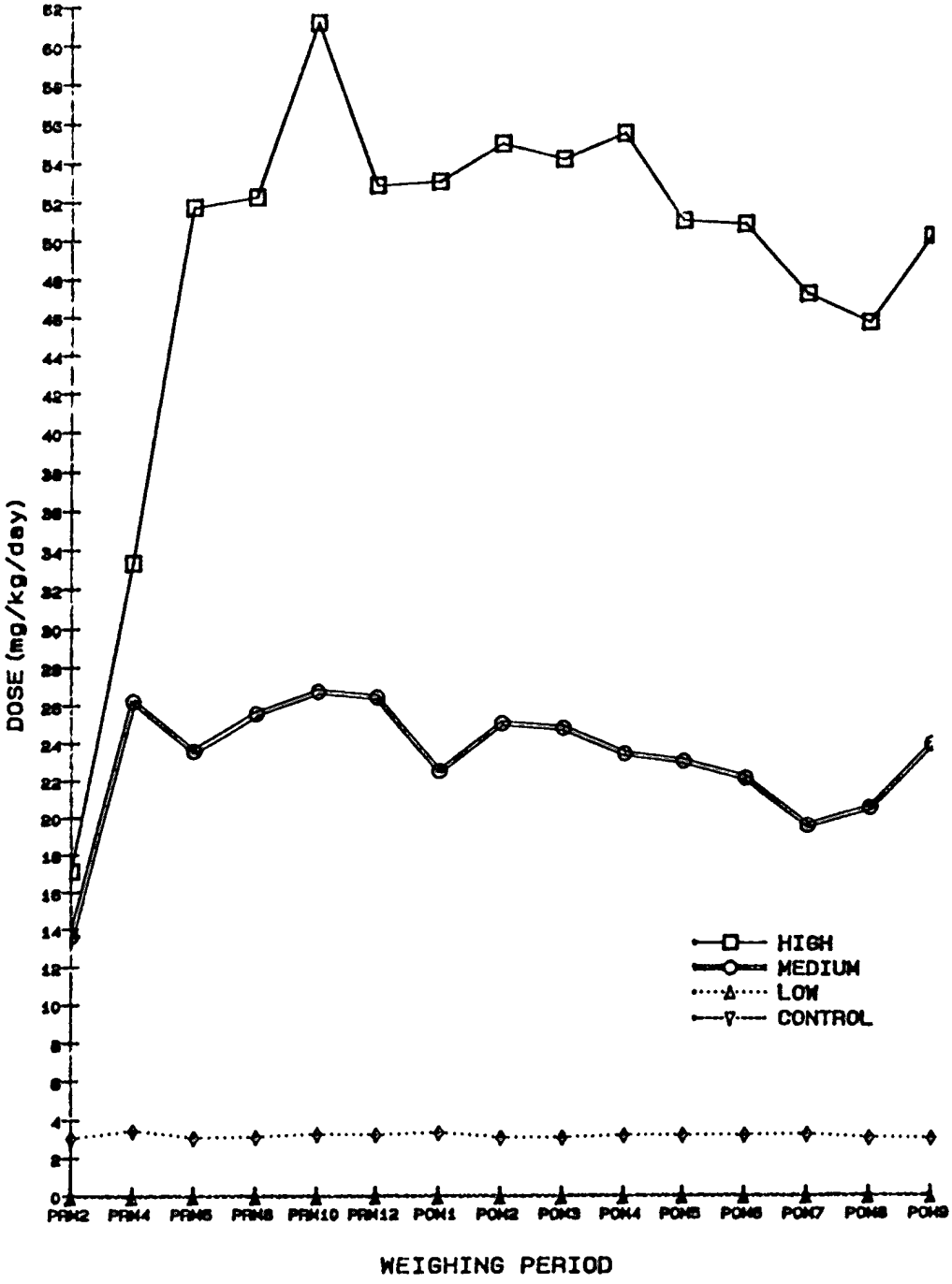


Figure 6. Mean Dose (mg TNB/kg/day) of Male Rats During Pre-mating and Post-mating Periods

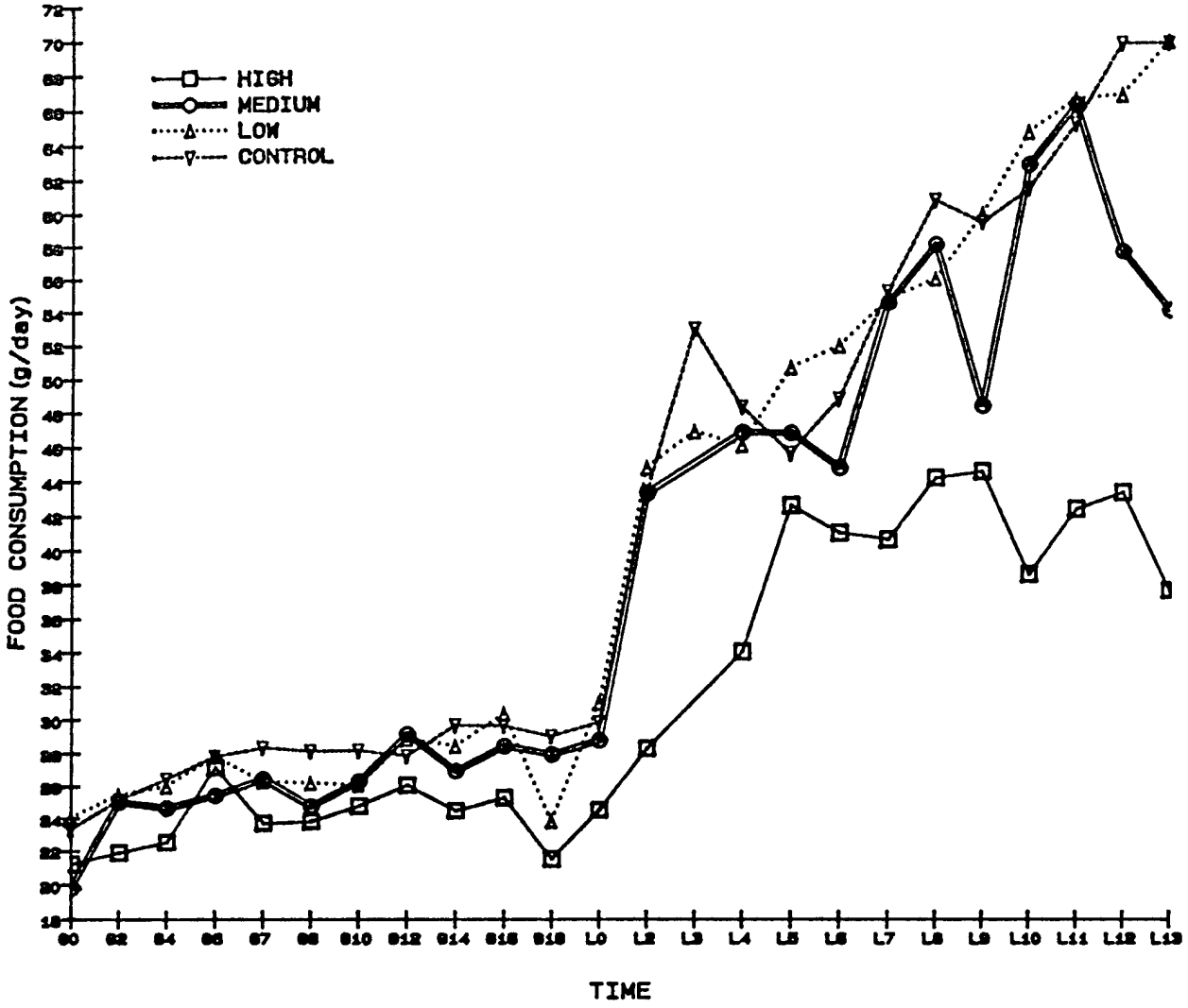


Figure 7. Mean Food Consumption of Female Rats During Gestation and Lactation Periods

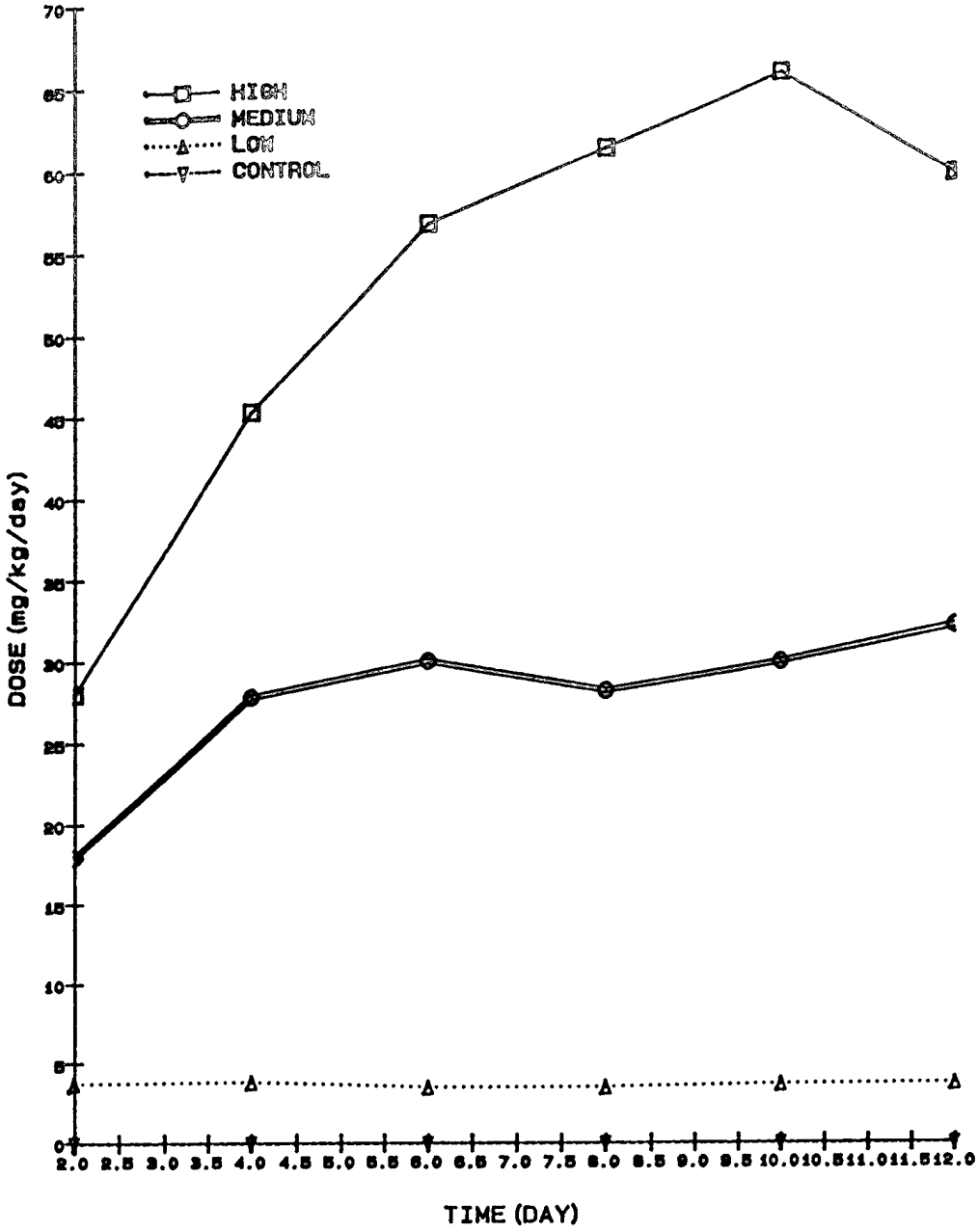


Figure 8. Mean Dose (mg TNB/kg/day) of Female Rats During the Pre-mating Period

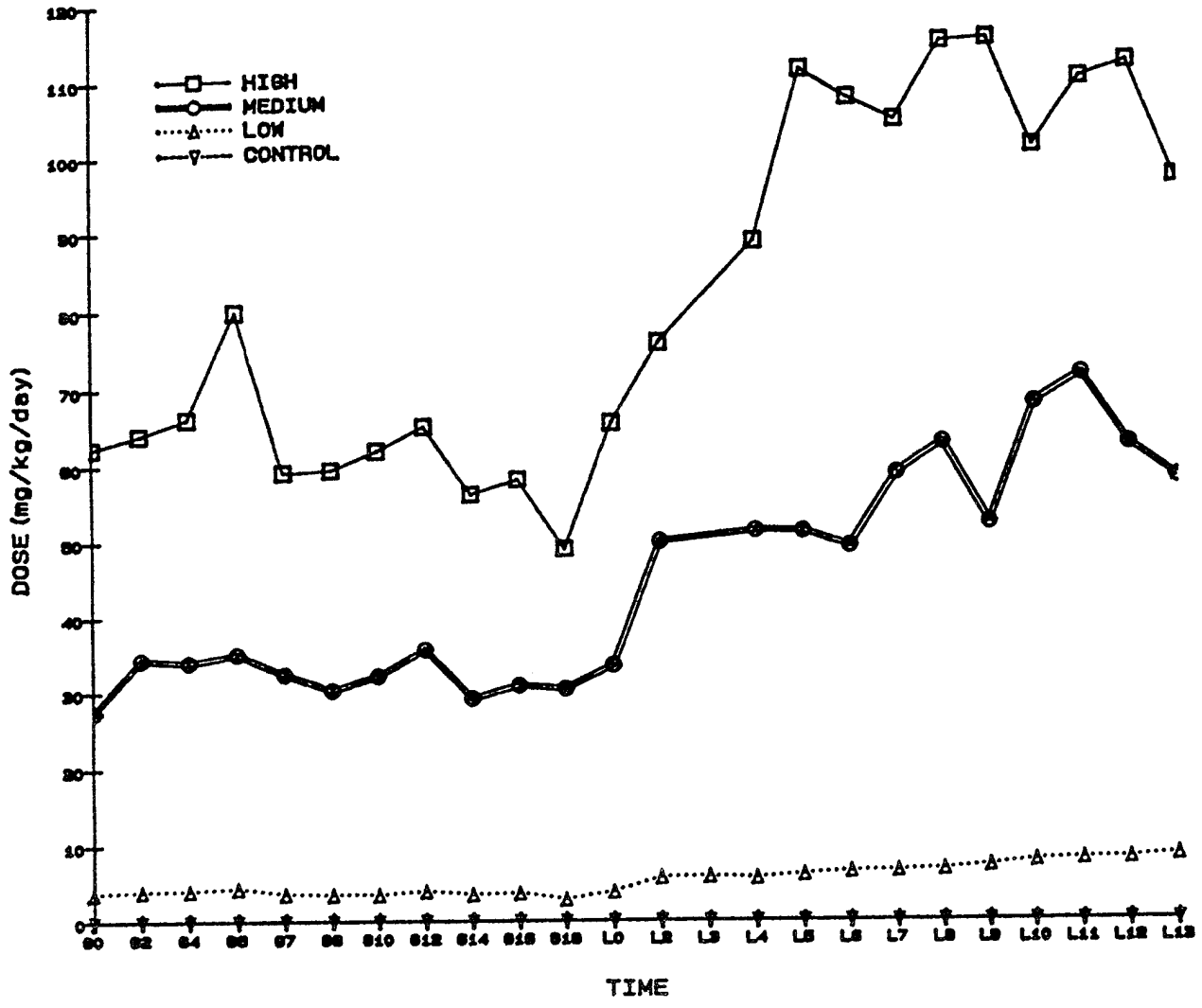


Figure 9. Mean Dose (mg TNB/kg/day) of Female Rats During Gestation and Lactation Periods

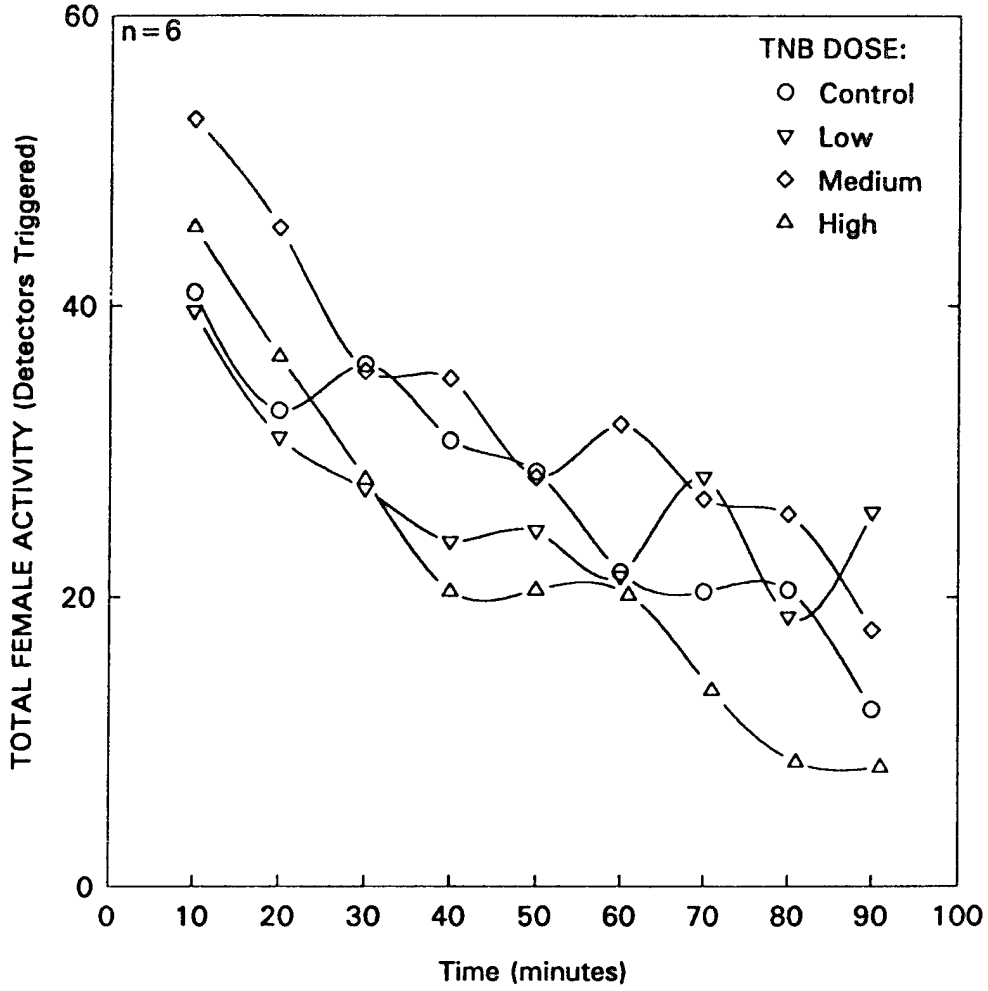


Figure 10. Figure-Eight Maze Activity of TNB-Treated and Control Rats at Study Termination

TABLE 1. SPERM EVALUATIONS FROM RATS ADMINISTERED TNB IN DIET

Parameter	Control (N=3)	Low (N=3)	Mid (N=2)	High (N=3)
Mean (±SD) Number Motile Cells Analyzed	280 ± 27	249 ± 59	153 ± 187	3 ± 4[a]
Concentration Motile (million/mL)	0.94	0.83	0.48	0.04[a]
Mean Number of Cells Traveling in a Circular Pattern	52 ± 4	38 ± 6	22 ± 30	0[a]
Percent Cells Traveling in a Circular Pattern	19	16	10	0[a]
Percent in Circular Pattern Compared to Total Cells	13	10	5	0[a]

[a]Different from control at $p < 0.05$.

TABLE 2. ABSOLUTE AND RELATIVE ORGAN WEIGHTS[a] OF MALE RATS TREATED WITH TNB

	Control	Low	Medium	High
Testes (g)	3.60 ± 0.11	3.61 ± 0.06	2.91 ± 0.25[b]	1.47 ± 0.06b
Ratio (%)	0.68 ± 0.02	0.68 ± 0.01	0.59 ± 0.05	0.32 ± 0.02b
Epididymides (g)	0.30 ± 0.02	0.24 ± 0.02	0.18 ± 0.05c	0.18 ± 0.01c
Ratio (%)	0.06 ± <0.01	0.04 ± <0.01	0.04 ± <0.01c	0.04 ± <0.01
Body Wt (g)	529.6 ± 5.56	528.0 ± 3.50	498.4 ± 9.61c	469.5 ± 10.17b

[a]Mean ± SEM, N=10.
[b]Statistically different from control at $p < 0.01$.
[c]Statistically different from control at $p < 0.05$.

TABLE 3. INCIDENCE SUMMARY OF SELECTED MICROSCOPIC LESIONS OF MALE RATS FOLLOWING ADMINISTRATION OF TNB IN DIET

		TNB Dose		
Organ/Lesion	Control	Low	Medium	High
Testes (N)	10	10	10	10
Degeneration	0	0	7[a]	10[a]
(severity)[b]	0	0	1.5[a]	2.8[a]
Epididymis (N)	10	10	10	10
Degenerated germ cells	0	0	8[a]	10[a]
Sperm Depletion	0	0	7[a]	10[a]
(severity)[c]	0	0	0.7[a]	4.1[a]
Brain (N)	2	2	2	2
Encephalitis, brain stem	0	0	0	1
(severity)[b]	0	0	0	1

[a]Statistically different from control at $p < 0.01$.
[b]Mean grades of severity based on 0=Normal; 1=Minimal; 2=Mild; 3=Moderate; 4=Marked; and 5=Severe.
[c]Mean grades of severity based on 2=Minimal (absence of sperm in the head and decreased sperm in the body of the epididymis); 3=Mild (absence of sperm in the head and body of the epididymis); 4=Moderate (absence of sperm in the head and body and decreased sperm density in the tail of the epididymis); 5=Severe (Absence of sperm in the epididymis).

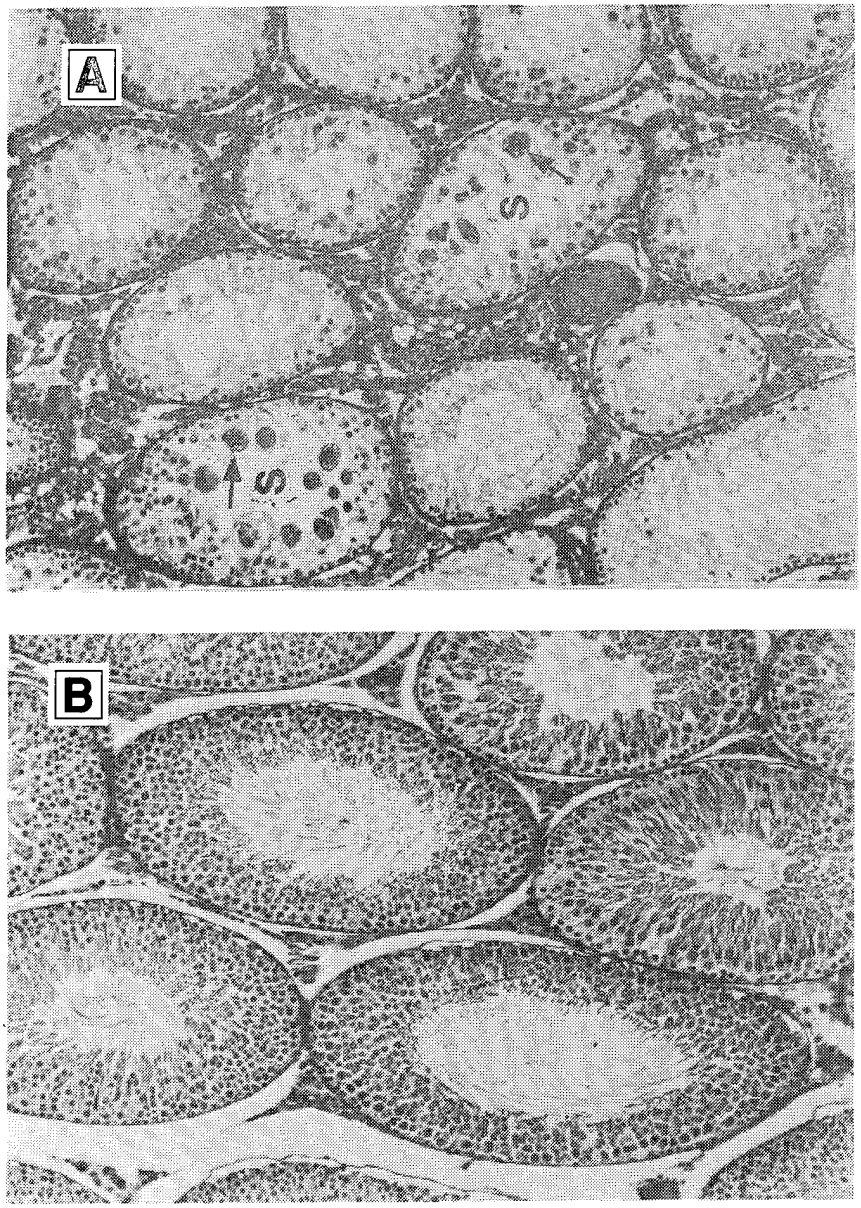


Figure 11. (A) Testis from a rat of the high-dose group showin marked degeneration and depletion for the germinal epithelium of the seminiferous tubules (S). Note giant cells composed of germ cell syncytia (arrows). PAS x120.
(B) Testis from a rat of the control group showin normal germinal epithelium lining the seminiferous tubules. PAS x120.

TABLE 4. INCIDENCE SUMMARY OF SELECTED MICROSCOPIC LESIONS OF FEMALE RATS FOLLOWING ADMINISTRATION OF TNB IN DIET

Organ/Lesion	Control	TNB Dose		
		Low	Medium	High
Spleen (N)	8	0	6	8
Hemosiderosis	0	–	6[a]	100[a]
(severity)[b]	0	–	3.8[a]	4.3[a]
Congestion	0	–	6[a]	7[a]
(severity)	0	–	2.2[a]	2.4[a]
Hematopoiesis	0	–	4[c]	6[c]
(severity)	0	–	0.8[c]	1.4[a]
Brain (N)	5	5	5	5
Encephalitis, brain stem	0	0	3[c]	5[c]
(severity)	0	0	1.4[c]	2.0[a]

[a]Statistically significant from control at $p < 0.01$.

[b]Mean grades of severity based on 0=Normal; 1=Minimal; 2=Mild; 3=Moderate; 4=Marked; and 5=Severe.

[c]Statistically significant from control at $p < 0.05$.

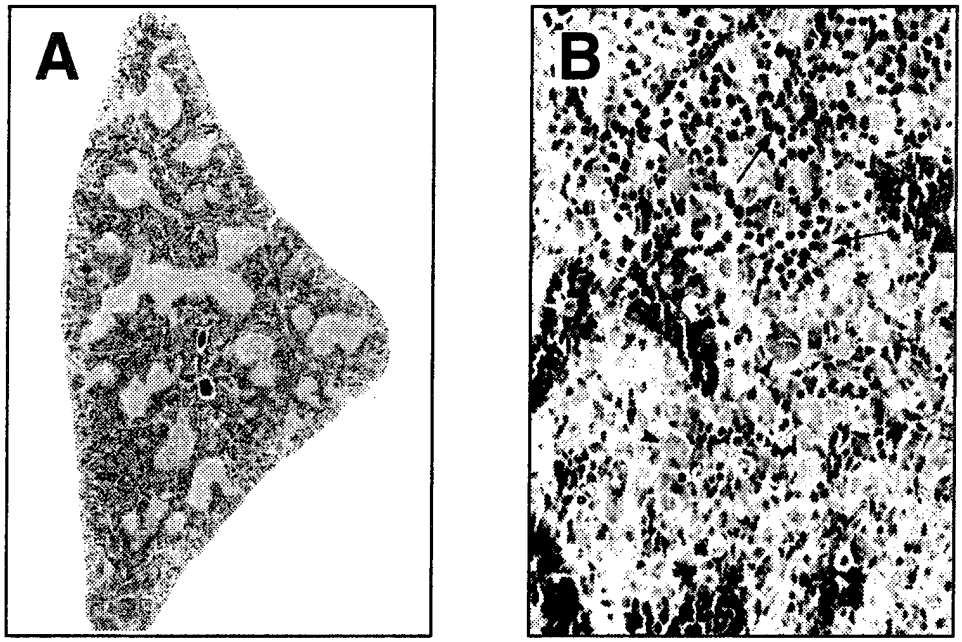


Figure 12. (A) Spleen from a rat of the high-dose group showing congestion of the red pulp. HE x8.5. (B) Higher magnification (x330) showing foci of erythrocytic extramedullary hematopoiesis (arrows) and numerous hemosiderin-laden macrophages (arrowheads).

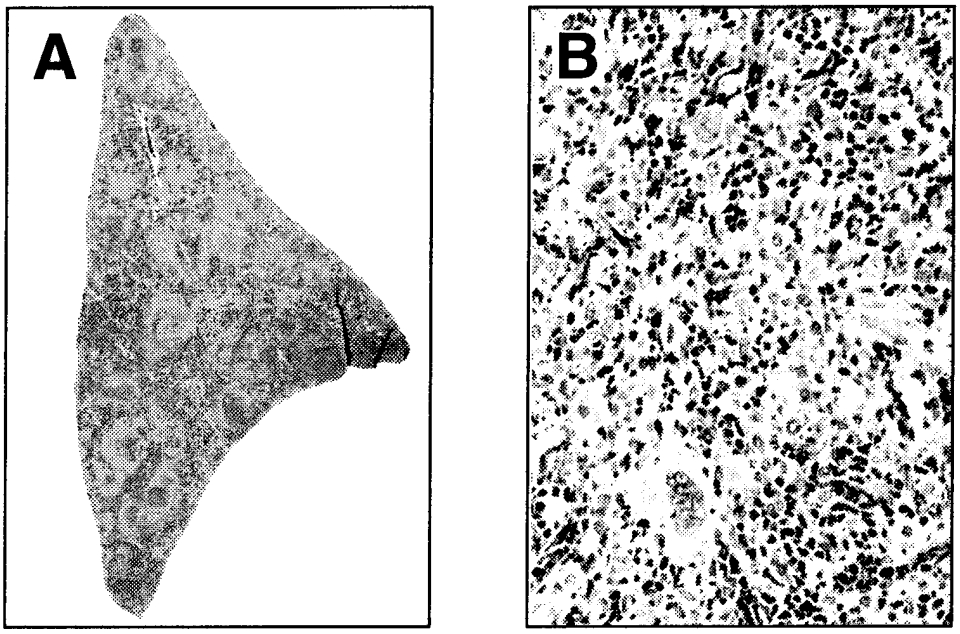


Figure 13. Spleen from a control rat. (A) HE x8.8 (B) HE x330

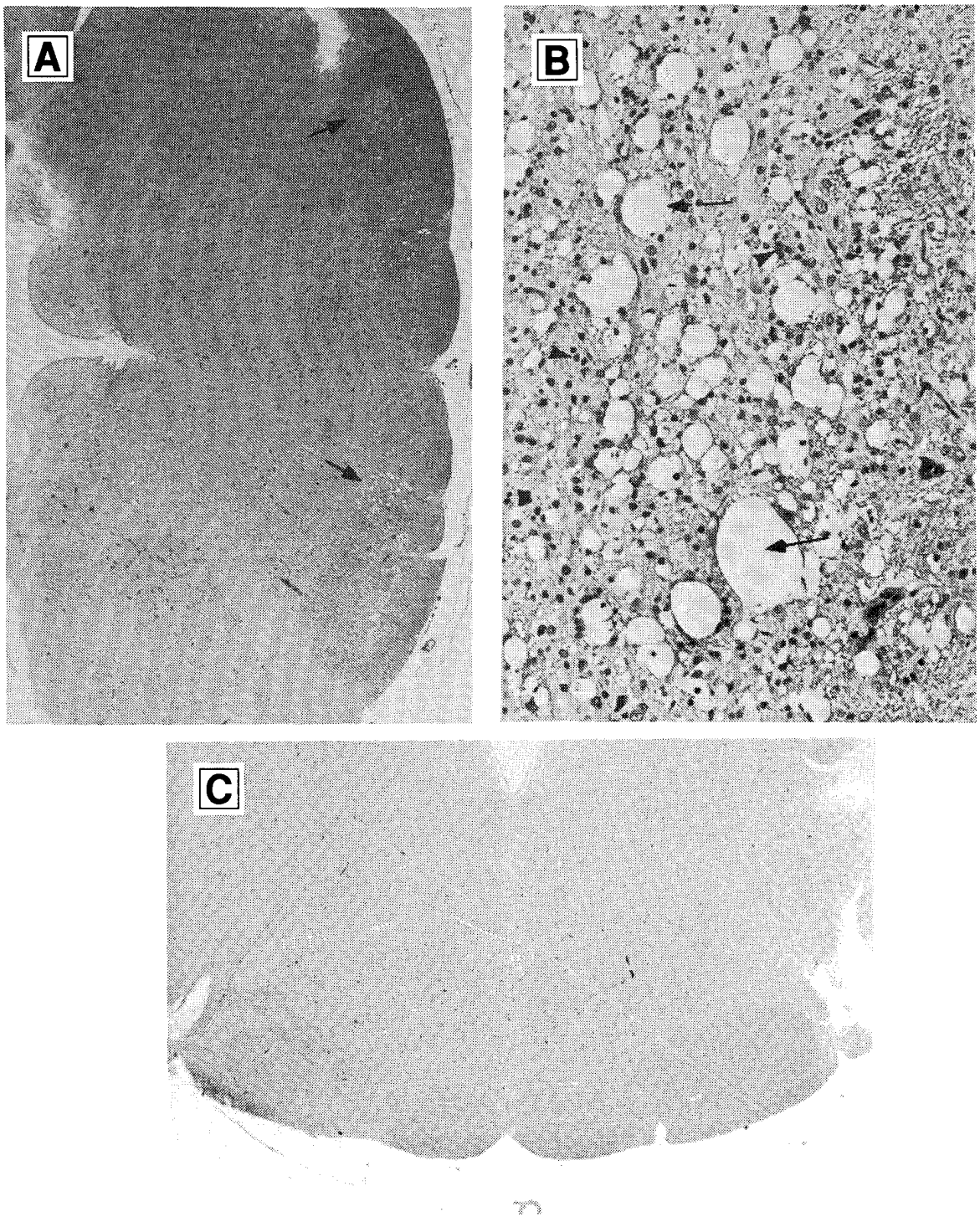


Figure 14. **(A) Medulla oblongata from a rat of the high-dose group showing bilateral rarefaction of the olivary region along the lateroventral brain stem (arrows). HE x15.**
(B) Higher magnification of the same area showing loss of large neuronal bodies of an olivary nucleus resulting in the prominent vacuolization of the neuropil (arrows). Also note gliosis (arrowheads). HE x150.
(C) Medulla oblongata of a control rat. HE x15.

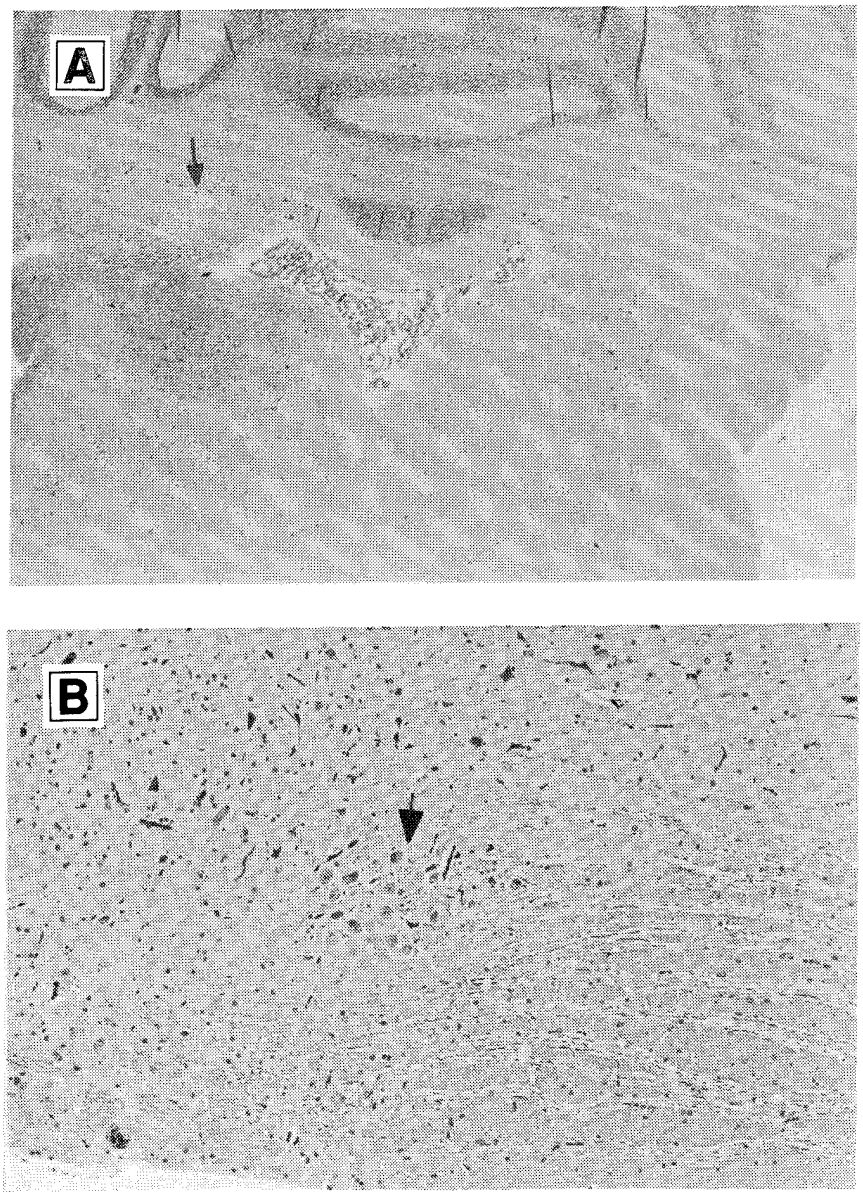


Figure 15. **(A) Cerebellar peduncle from a rat of the mid-dose group. Unilateral rarefaction of the region of the deep cerebellar nucleus (arrow) of the peduncle. HE x15.**
(B) Higher magnification of the medulla oblongata from a control rat. Note the large olivary neurons (arrow). HE x30.

TABLE 5. LITTER DATA FOR RATS TREATED WITH TNB

		TNB Dose		
	Control	Low	Medium	High
No. of mated pairs	10	10	10	10
No. of copulated pair	10	10	10	10
No. of dams with pups born	10	9	9	10
No. of dams with pups alive	10	9	9	10
Gestation index (%)[a]	100.0	90.0	90.0	100.0
Live birth index (%)[b]	98.8	98.5	09.6	03.8
4-Day survival index (%)	98.9	91.0	96.6	86.1[c]
7-Day survival index (%)	100.0	100.0	100.0	94.9
14-Day survival index (%)	98.7	100.0	98.6	100.0
21-Day survival index (%)	100.0	100.0	100.0	86.7[c]
Lactation index (%)[d]	98.7	100.0	98.6	82.3[c]

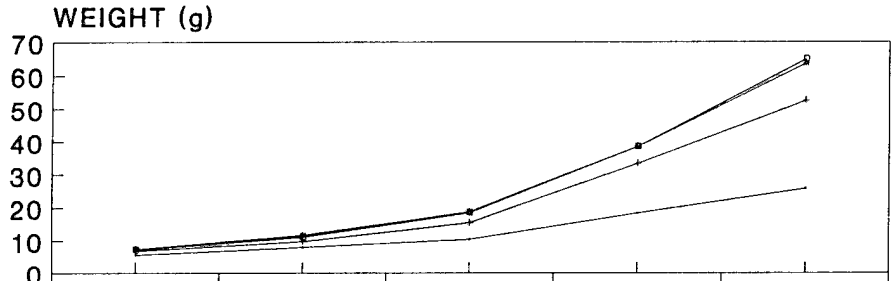
[a] $\frac{\text{Number of females with live litters}}{\text{Number of females pregnant}}$

[b] $\frac{\text{Number of live pups at birth}}{\text{Total number of pups born}}$

[c] Statistically different from control at $p < 0.05$.

[d] $\frac{\text{Number of pups surviving 21 days}}{\text{Total number of live pups at 4 days}}$

A02-RF MALE RAT PUPS
LITTER MEAN BODY WEIGHTS

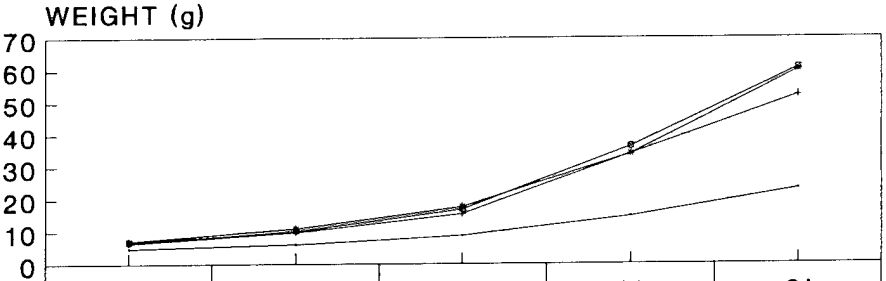


	1	4	7	14	21
HIGH	5.7	8	10.4	18.4	25.7
MEDIUM	6.9	9.7	15.4	33.4	52.5
LOW	7.4	11.6	18.7	38.6	63.7
CONTROL	7.3	11.1	18.5	38.6	64.9

DAY OF EXPOSURE

HIGH MEDIUM LOW CONTROL

A02-RF FEMALE RAT PUPS
LITTER MEAN BODY WEIGHTS



	1	4	7	14	21
HIGH	4.8	6	8.74	14.7	23.1
MEDIUM	6.5	9.7	15.5	34	52.2
LOW	7.2	10.9	17.6	34	60.0
CONTROL	6.8	10.1	16.9	36.4	60.6

DAY OF EXPOSURE

HIGH MEDIUM LOW CONTROL

Figure 16. Male and Female Pup Mean Body Weights

SECTION 4
SUMMARY

Effects noted during and following the study:

High-Dose TNB Animals

Body Weight Effects. Male and female mean body weights were significantly ($p < 0.01$) decreased when compared to control animals. Mean body weights were decreased by 11 and 15% when compared to control mean body weights in the male and female rats, respectively, at sacrifice.

Clinical Signs and Neuropathology. 100% of dams showed clinical signs of altered locomotor activity starting postpartum and continuing through necropsy. Brain lesions were noted in 100% of dams examined at study termination. Similar brain lesions were noted in one of two male rats examined following 35 days of treatment. The male rat with brain lesions did not show any clinical signs of altered locomotor activity during the study.

Sperm Morphology. Adverse effects in sperm motility and concentration.

Male Reproductive Organ Weights and Pathology. Testes ($p < 0.01$) and epididymides ($p < 0.05$) weights were significantly less than control values. Ratios of organ-to-body weights were significantly different from controls. Degeneration of the testes and epididymides was observed during the histopathological examination.

Splenomegaly (females). Discoloration and enlargement of spleens in females rats were observed. The incidence of congestion, hemosiderosis, and EMH was also increased.

Locomotor Tests. In the female rats, distance traveled and ambulatory time were significantly ($p < 0.05$) increased in the Opto-Varimex test.

Reproductive Indices. Reproductive indices were not different from control. Pup survival indices were decreased at 4 and 21 days postpartum.

Pup Weights. Pup mean body weights were decreased ($p < 0.01$).

Mid-Dose Animals

Body Weight Effects. Male and female mean body weights were significantly ($p < 0.01$) less than control animals.

Clinical Signs and Neuropathology. Only a transitory effect in one female rat (head tilt). Circling noted in some rats. Brain lesions noted in 60% of dams examined at study termination.

Splenomegaly (females). Discoloration and enlargement of spleens were observed. The incidence of congestion, hemosiderosis, and EMH were also increased.

Male Reproductive Organ Weights and Pathology. Testes and epididymides weights were significantly less than control values. Degeneration of these organs was confirmed during the histopathological examination.

Locomotor Tests. No differences from control animals were noted in both behavioral tests.

Reproductive Indices. No differences from control animals were noted in reproductive indices or survival indices.

Pup Weights. Male pup weights were significantly different at days 14 and 21; female pups at day 21 exhibited weight loss.

Low-Dose Animals

Body Weights. Mean body weights of male rats were similar to control weights, while female rats were approximately 7% less than control values, but were not statistically significant.

Clinical Signs and Neuropathology. Circling was noted in some rats during the treatment period. No neural lesions were noted during histopathologic examination.

SECTION 5
DISCUSSION

Reproductive Toxicity

Treatment with TNB in the diet of male and female Sprague-Dawley rats did not produce any adverse effects on the reproductive performance of the male rats even though lesions of the reproductive system were observed. The majority of the seminiferous tubules had marked (or less) alterations in rats of the high- and mid-dose groups. Because spermatogonia were present in most altered tubules, recovery of sperm production would have been likely if the TNB exposure regimen had been terminated after 35 days of treatment.

Spleen Toxicity

Collectively, the splenic lesions strongly suggest a TNB-induced dyscrasia of erythrocytes, consistent with regenerative anemia.

Neurotoxicity

All rats that displayed clinical signs of altered locomotion had brain lesions. However, brain lesions were noted in two mid-dose female rats and one high-dose male rat that did not display clinical signs. The distribution and spongiform pattern of the vacuolated neuropil suggest a loss of large neurons in the olivary, deep cerebellar, and vestibular nuclei. Such effects would likely produce the observed clinical manifestations of neurologic disease. However, the distribution (site specific), nature, and extent of the brain lesion should not be over-interpreted in this range-finding study. Specific distribution and development of the brain lesions induced by TNB would best be studied with a time-course experimental design with perfusion fixation and extensive systematic sampling of the brain.

The purpose of this study was to assist in selecting dose levels for a 90-day modified SIDS reproductive study. The dose levels selected for the 90-day SIDS study were 300, 150, and 30 mg TNB/kg diet which would result in target doses of 25, 12.5, and 2.5 mg/kg/day, respectively. These treatment dose selections should provide a high dose that produces toxicity (testis and sperm effects in male rats; neurotoxic effects in female rats), but not mortality. The selected mid dose is approximately one-half the range-finder mid dose and is expected to produce minimal observable toxic effects. The lowest level is not expected to produce toxicity. Hematology and bone marrow assessments will be done

on the TNB-treated animals. Additional males will be included in the 90-day SIDS study to determine recovery from any male reproductive effects that may be observed during TNB treatment.

SECTION 6

REFERENCES

Barcikowski, R.S., ed. 1983. *Computer Packages and Research Design.* Lanham, MD: University Press of America.

Cody, T.E., S. Witherup, L. Hastings, K. Stemmer, and R.T. Christian. 1981. 1,3-Dinitrobenzene: Toxic effect *in vivo* and *in vitro*. *J. Toxicol. Environ. Health* **7**(5):829-847.

Evelyn, K.A. and H.T. Mallog. 1938. Microdetermination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. *J. Biol. Chem.* **126**:655-662.

Fitzgerald, C.B., N. DiGiulio, L.S. Desai, and G. Reddy. 1992. Acute toxicological evaluation of 1,3,5-Trinitrobenzene. *Acute Toxicity Data* **1**(3):169-170.

Furedi, E.M., B.S. Levine, D.E. Gordon, V.S. Rac, and P.M. Lish. 1984a. *Determination of the chronic mammalian toxicologic effects of TNT (Twenty-four month chronic toxicity/carcinogenicity study of trinitrotoluene (TNT) in the Fischer 344 rat).* Final Report - Phase III, Vol. 1. IIT Research Institute, Project No. L6116, Study No. 11, Chicago, IL. DAMD17-79-C-9120. AD-A168 754.

Furedi, E.M., B.S. Levine, J.W. Sagartz, V.S. Rac, and P.M. Lish. 1984b. *Determination of the chronic mammalian toxicologic effects of TNT (Twenty-four month chronic toxicity/carcinogenicity study of trinitrotoluene (TNT) in the B6C3F$_1$ hybrid mouse).* Final Report - Phase IV, Vol. 1. IIT Research Institute, Project No. L6116, Study No. 11, Chicago, IL. DAMD17-79-C-9120. AD-A168 754.

Hathaway, J.A. 1977. Trinitrotoluene: A review of reported dose-related effects providing documentation for a workplace standard. *J. Occup. Med.* **19**(5):341-345.

Levine, B.S., J.H. Rust, J.M. Burns, and P.M. Lish. 1983. *Determination of the chronic mammalian toxicological effects of TNT (Twenty-six week subchronic oral toxicity study of trinitrotoluene (TNT) in the beagle dog).* Phase II, Final Report, IIT Research Institute, Report No. L6116, Study No. 5, Chicago, IL. DAMD 17-79-C-9120, AD-A157 082.

Levine, B.S., E.M. Furedi, D.E. Gordon, P.M. Lish, and J.J. Barkely. 1984. Subchronic toxicity of trinitrotoluene in Fischer 344 rats. *Toxicology* **32**:253-265.

Morton, A.R., M.V. Ranadive, and J.A. Hathaway. 1976. Biological effects of trinitrotoluene from exposure below the threshold limit value. *Am. Ind. Hyg. Assoc. J.* **37**(1):56-60.

Rosner, B. 1990. Fundamentals of Biostatistics. Boston, MA.: PWS-Kent Publishing Co.

Ryon, M.G., B.C. Pal, S.S. Talmage, and R.H. Ross. 1984. *Database assessment of the health and environmental effects of munition production waste products.* Final Report. ORNL-6018. (NTIS DE84-016512). Oak Ridge National Laboratory, New York, NY, p. 3403.

Sokal, R.R. and F.J. Rohlf. 1981. *Biometry*, Second Edition, W.W. Freeman and Company, New York, NY.

Stewart, A., L.T. Witts, G. Higgins, et al. 1945. Some early effects of exposure to trinitrotoluene. *Br. J. Ind. Med.* **2**:74-82. (Cited in Hathaway, 1977)

U.S. EPA. 1989. *Health and Environmental Effects Profile for 1,3,5-Trinitrobenzene*. Environmental Criteria and Assessment Office, Office of Health and Environmental Assessment, Cincinnati, OH, ECAO-CIN-G071.

APPENDIX A
DIET PREPARATION AND ANALYSIS

Diet

1,3,5-Trinitrobenzene powder (CAS 399-35-4) was supplied by the U.S. Army Biomedical Research and Development Laboratory. Analysis by HPLC revealed no detectable impurities. Certified powdered Purina Laboratory Chow 5002 was purchased (Ralston Purina Co., St. Louis, MO) and stored at 4 °C until used. 1,3,5-Trinitrobenzene diets were prepared as needed. First, 1.2 g of TNB was added to 50 g of powdered diet in a mortar and thoroughly ground with a pestle. Afterwards, 200 g of the diet was added and mixed for 15 min followed by 550 g and mixed for an additional 15 min. Finally, the remaining diet (700 g) was added and mixed for 30 min in a mechanical mixer (Kitchen Aid, St. Joseph, MI) for uniform distribution of TNB in the diet. This was verified by determining the TNB concentration in the diet, taken from each of the 1 kg mixtures, by quantitative analysis done by HPLC. The premixed diet (0.8 g/kg) was further diluted with fresh powdered diet to obtain the desired TNB concentration in the lower dose groups. The diet feeders were changed twice a week.

Diet Analysis

Analyses of the TNB-feed mixtures were carried out on acetone extracts of the mixtures, utilizing a Waters 600E chromatography system (Waters, Milford, MA), equipped with a 490E programmable multiwavelength detector, operating at 254 nm. The entire chromatography system was interfaced with a Berthold HPLC computer program, Version 1.65 (Berthold, Nashua, NH). The TNB was eluted from a Zorbax C-8 column (9.4 mm x 25 cm) (MAC-DOD Analytical, Chadds Ford, PA) with a water-methanol gradient, at a flow rate of 3 mL/min. Working standards were prepared in Burdick and Jackson HPLC grade high purity methanol (Baxter, Obetz, OH).

U. S. Government Printing Office 1995 650-075/00067